# Antisense Research and Applications

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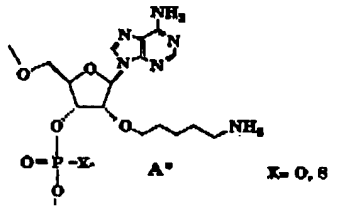


SCHEME 9. The ISIS 2′-aminolinker.

## III. SUGAR MODIFICATIONS

As discussed in Section II, in post-oligonucleotide synthesis, a nucleophile such as an amino group with an appropriate linker can be introduced at the 3′ or 5′ end of the oligonucleotide, at the 5-position of uracil, $N^4$ position of cytosine, and $N^6$, $N^2$ positions of purine, as well as in the phosphodiester backbone. Each of these approaches, however, has limitations. The terminal linkers place the functional groups at the ends and thus limit recognition of a given site within the double helix. Linkers attached to the bases may interfere with base pairing and/or stacking interactions, and linkers attached to the backbone present chirality problems. At ISIS Pharmaceuticals we have developed conjugation chemistry suitable for both DNA and RNA modifications that is based on an aminolinker (2′-*O*-pentylamine) attached to the 2′-*O*-position of the sugar.

### A. CONJUGATION AT THE 2′ POSITION

#### 1. Chemistry

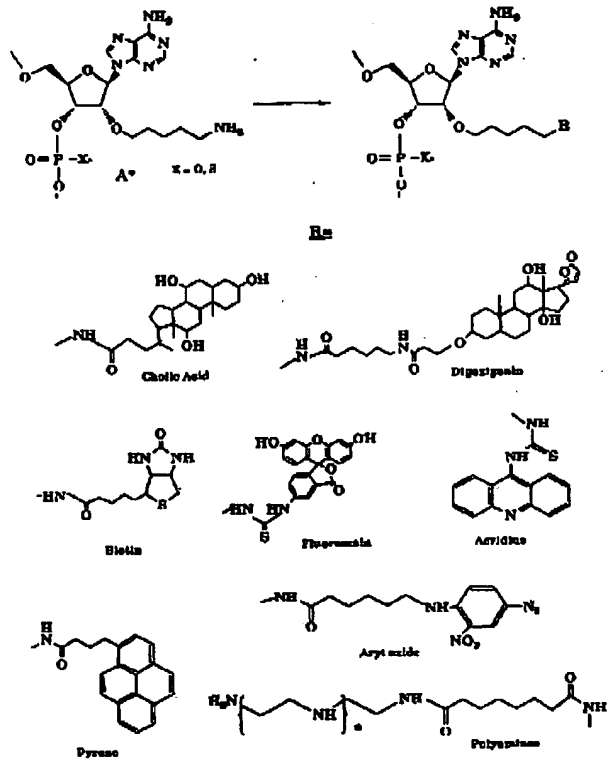
The chemistry for constructing a phosphoramidite monomer with a 2′-aminolinker is based on alkylation reactions of adenosine developed by Guinosso and Cook.[91] The monomer, which is designed for automated DNA synthesis, is produced by alkylation of the anion resulting from NaH/DMF treatment of adenosine at 0 to 5°C with N-(5-bromopentyl)phthalimide base protection with benzoyl chloride employing transient protection of 5′- and 3′-hydroxyls followed by tritylation and phosphitylation to obtain the desired phosphoramidite.

Using this 2′-aminolinker, we have conjugated various molecules[92] to oligonucleotides: (1) cholic acid, for uptake enhancement; (2) digoxigenin, a steroidal molecule that has hydrophobic properties that enhance uptake, and it is also a reporter molecule in a commercially available detection system; (3) biotin; (4) fluorescein, which are reported molecules to study uptake; (5) pyrene; (6) acridine intercalators; (7) aryl azides which are photoactivatable crosslinking agents; and (8) polyamines such as spermine and pentaethylenehexamine for uptake studies and also as potential cleaving molecules.

Shown below are the oligodeoxynucleotides I and II, which incorporate the 2′-*O*-modified adenosine (indicated as A*). The sequence belongs to the E2 region of the bovine papilloma virus-1 (BPV-1) and has demonstrated an antisense effect. The nucleoside compositions of I and II were established by HPLC analysis after the oligonucleosides had been cleaved by snake venom phosphodiesterase and calf-thymus alkaline phosphatase.

I: 5′CTGTCTCCA*TCCTCTTCACT3′
BPV sequence, single-site labeling

II: 5′CTGTCTCCA*TCCTCTTCA*CT3′
BPV, double-site labeling



**SCHEME 10.** Conjugations at the 2′ position.

Oligonucleotides I and II were reacted with the compounds summarized in Scheme 10, each of which had a functional group reactive to an amino group. Each of the functionalities mentioned above was conjugated to the oligomers. The conjugation reactions were carried out in an aqueous buffer at pH 8 to 9 under standard conditions. The conjugation yields varied between 70 and 90%. This approach facilitates multiple conjugation; a product with multiple labels could be synthesized and purified by HPLC. In addition, we have synthesized oligonucleotides containing a phosphorothioate backbone and RNA analogs with 2′-OMe groups that incorporate our 2′-aminolinker at a specific site. These derivatives are already known to have either nuclease stability (thioates) or enhanced hybridization properties (2′-OMe derivatives). Conjugations to the 2′-aminolinker were carried out from thioates and RNA mimics as well. The 2′-aminolinker provides an additional handle for conjugating other functionalities, such as lipophilic groups to improve membrane transport properties or nucleic acid cleaving agents.

## 2. Biophysical Studies

### *a. Thermal Melt Analysis*

First, the effect of the 2′-aminolinker alone was studied;[93] a 17-mer oligonucleotide (GGA*CCGGA*A*GGTA*CGA*G) incorporating five 2′-*O*-aminopentoxy modifications was synthesized and purified. On melting against DNA, a net destabilization of 6.1°C was observed which averages to −1.2°C/modification. Against RNA, at the same time, a net stabilization of 1.1°C was noted which translates to 0.22°C stabilization/modification. Similarly the ISIS 1570 oligonucleotide phosphorothioate (TGGGA*GCCA*TA*CGA*GGC)

**TABLE 2**
**Duplex Melting Temperature of the 2′-Conjugates of the BPV Oligonucleotide (Against DNA)**

I: 5′-CTG TCT CCA*TCC TCT TCA CT-3′
II: 5′-CTG TCT CCA*TCC TCT TCA*CT-3′
III: 5′-CTG TCT CCA TCC TCT TCA CT-3′

| Oligo | Modification | $T_m$ °C[a] | $\Delta T_m$/mod[b] |
|---|---|---|---|
| III | Wild type | 60.5 | — |
| I | 2′-*O*-Pentyl-$NH_2$(1 mod) | 58.1 | — |
| IB | Biotin conjugate | 56.4 | −1.7 |
| IC | Cholic acid conjugate | 55.5 | −2.6 |
| ID | Digoxigenin conjugate | 55.8 | −2.3 |
| IF | Fluorescein conjugate | 55.1 | −3.0 |
| IP | Pyrene conjugate | 62.6 | +4.5 |
| IA | Acridine conjugate | 58.6 | +0.5 |
| II | 2′-*O*-Pentyl-$NH_2$(2 mod) | 56.9 | — |
| IIB | Biotin conjugate | 54.4 | −1.3 |
| IIC | Cholic acid conjugate | 54.3 | −1.3 |
| IID | Digoxigenin conjugate | 53.8 | −1.6 |
| IIF | Fluorescein conjugate | 53.4 | −1.8 |
| IIP | Pyrene conjugate | 65.1 | +4.1 |
| IIA | Acridine conjugate | 58.1 | +1.2 |

[a] $T_m$ buffer used = 100 m*M* NaCl, 10 m*M* $Na_2PO_4$, 0.1 m*M* EDTA, pH 7.0.
[b] Compared to the modified Oligo I or II as appropriate.

was synthesized replacing all four adenosines with the aminolinker containing adenosine. The resultant 18-mer oligonucleotide had the same $T_m$ as the parent thioate against the RNA complementary strand. Thus, in antisense applications, a 2′-aminolinker does not affect duplex hybridization and even offers some small stabilization.

Second, the conjugates from oligonucleotides I and II were studied (Table 2). In thermal melting studies[94] against complementary DNA, we have observed nearly 2 to 3°C destabilization for substituents like biotin, fluorescein, digoxigenin, and cholic acid. Even large steroidal molecules exhibit only modest destabilization. The destabilization observed is less pronounced than for the base and backbone modifications mentioned earlier. Furthermore, the destabilizing effects are *not* additive: $\Delta T_m$/modification is less for doubly conjugated oligonucleotides than for singly modified oligonucleotides.

In the case of pyrene and acridine conjugates, enhanced duplex stability has been observed. The stabilization was significant in pyrene conjugates (4°C/modification) and marginal in the case of acridine conjugates (0.5 to 1°C/modification), although this difference may be due to different lengths of the groups involved between acridine and the aminolinker used vs. pyrene and the aminolinker (longer linker in the second case). We are pursuing NMR and other spectroscopic (fluorescence quenching) studies to confirm intercalation of these ligands in duplexes.

In the cases of pyrene and fluorescein modifications in single strand oligodeoxynucleotides, fluorescence properties were found to be additive; in single strand conjugates like the one derived from oligonucleotide II shown above, there was no fluorescence quenching of one chromophore by the other.

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# Antisense Drug Technology

## Principles, Strategies, and Applications

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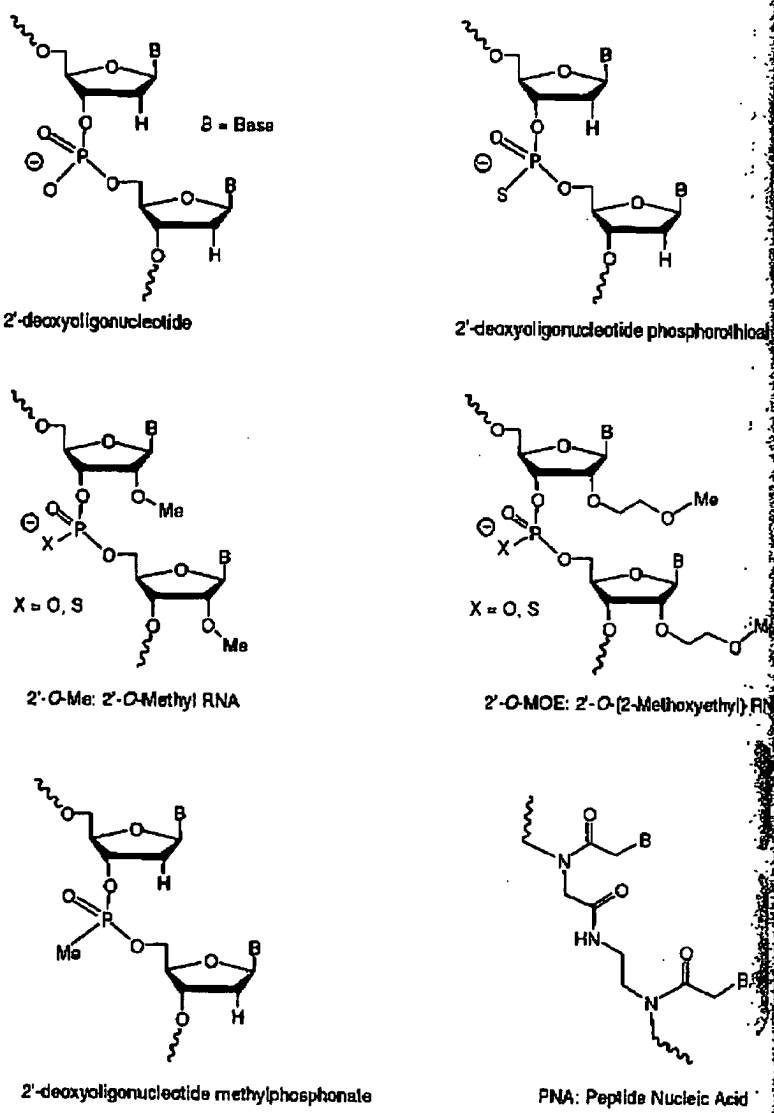



**Figure 2** First-generation and second-generation chemistries to which ligands have been conjugated.



philicity dominates due to the extensive hydrogen bonding possible with the phosphate and sugar residues. This intrinsic hydrophilicity is augmented by the anionic nature of the backbone. The hydrophilic character and the anionic backbone of the drug reduces cellular permeation. Conjugation of lipophilic molecules is the obvious way to solve the cellular permeation problem.

Various lipophilic molecules have been conjugated to antisense oligonucleotides and Fig. 3 shows the structures of the compounds. Among them, cholesterol is perhaps the best characterized. It has been studied by various groups for the past 11 years (11) and has been reported to enhance binding of oligonucleotides to lipoproteins and, thereby, enhance cellular association and transport (12,13). The majority of this section will concentrate on the considerable data available on cholesterol-conjugated oligonucleotides. Data available on other lipophilic ligands will also be summarized.

## 2. Uridine-Conjugated Lipophilic Phosphoramidites and Solid Supports

Synthesis of 5′-*O*-dimethoxytrityl-2′-*O*-(6-aminohexyl)uridine and the 3′-isomer, 5′-*O*-dimethoxytrityl-3′-*O*-(6-aminohexyl)uridine, has been described by Manoharan et al. (14,15). Derivitization of these amines (Fig. 4) with cholesteryl chloroformate yielded cholesterol carbamate derivatives. Adamantane acetic acid, eicosenoic acid, and pyrene butyric acid were converted to their pentafluorophenol esters and condensed with these amines. 1,2-Di-*O*-hexadecyl-rac-glycerol was converted to the corresponding carbonate using disuccinimidyl carbonate. The carbonate was condensed with the amines to yield the modified nucleosides containing linkages. The nucleoside conjugates, after purification on a silica gel column, were phosphitylated to yield the corresponding phosphoramidites and then incorporated into oligomers. Each nucleoside was then condensed with long-chain alkylamino controlled pore glass (CPG).

## 3. Cholesterol-Conjugated ICAM-1 Antisense Oligonucleotides

An antisense oligonucleotide targeting the 3′ untranslated region of mouse intercellular adhesion molecule-1 (ICAM-1) was used for characterization of lipophilic conjugates. ISIS-3082 (see Table 1 for oligonucleotide sequences), a phosphorothioate oligonucleotide, shows antisense inhibition in cell culture with an $IC_{50}$ of 100 nM when formulated with a cationic lipid for delivery. Uridine nucleoside synthons containing cholesterol at the 2′ or 3′ position were synthesized and incorporated at the 5′ end of the ISIS-3082 resulting in the oligonucleotide-cholesterol conjugate ISIS-8005 (14).

Cell culture experiments were used to evaluate the effect of ISIS-3082 and ISIS-8005 on ICAM-1 expression without any cationic lipid. ISIS-8005 inhibited ICAM-1 in a dose-dependent manner with an $IC_{50}$ of 2.5 μM, while ISIS-3082

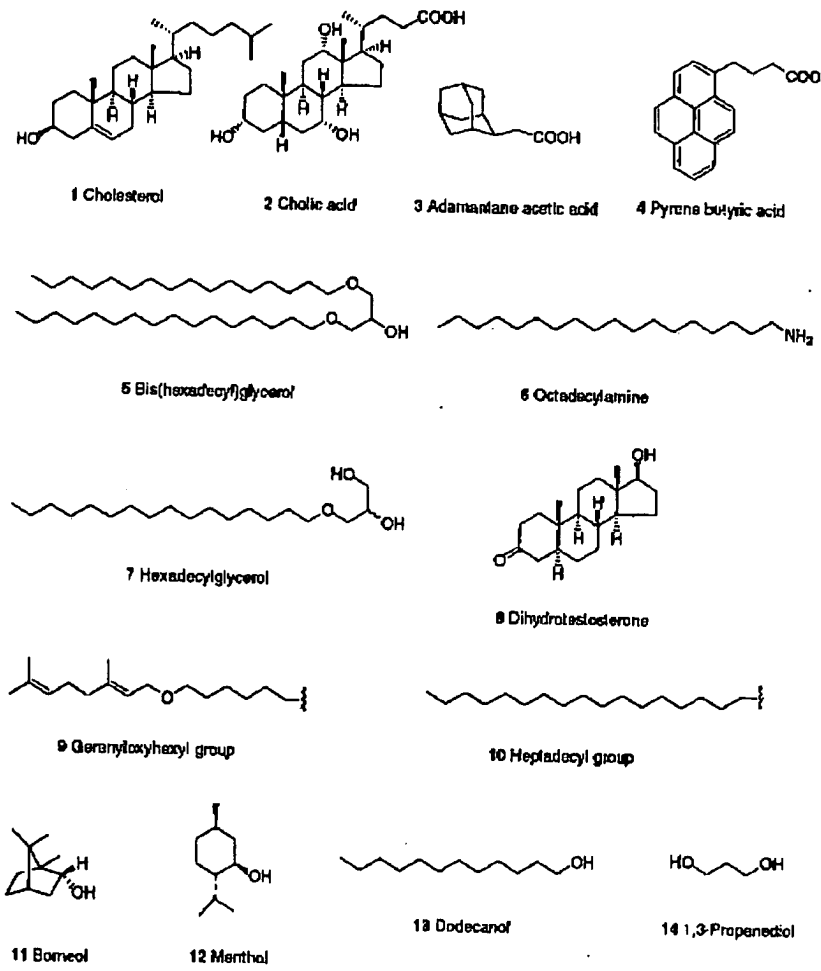



**Figure 3** Lipophilic molecules.



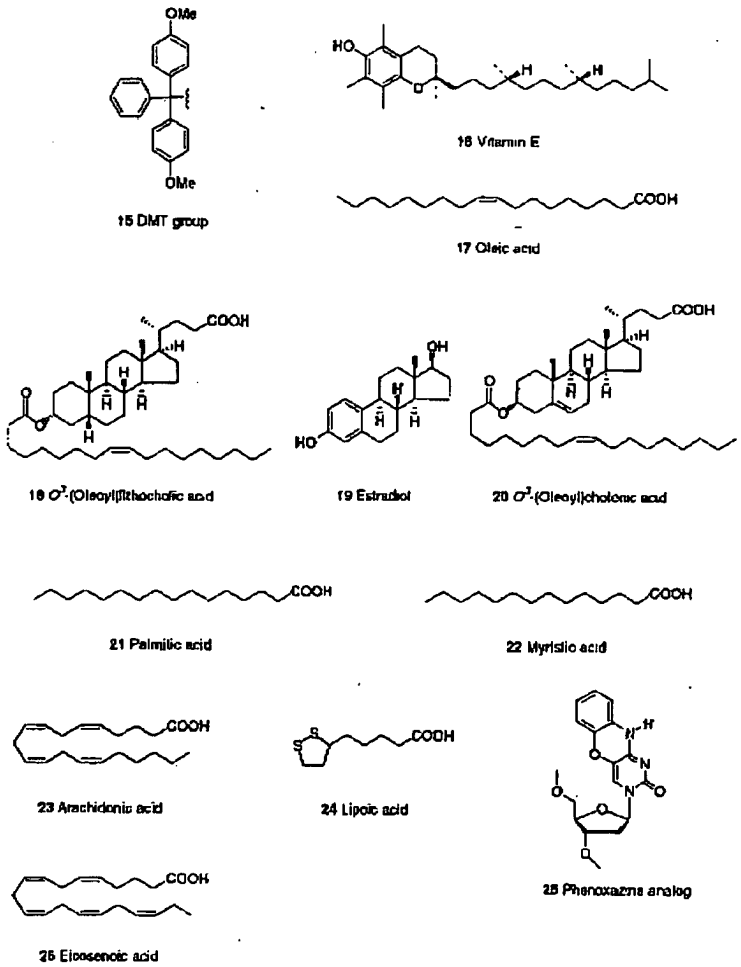



**Figure 3** Continued

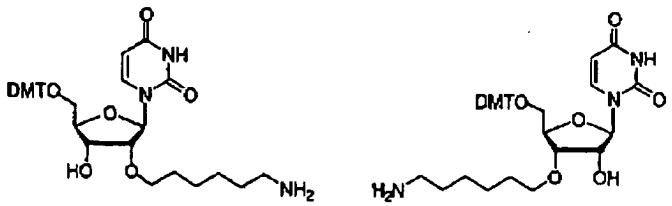



**Figure 4** 2′- and 3′-*O*-(6-aminohexyl) uridine derivatives.

did not show any activity, even when high concentrations of oligonucleotide were used. Furthermore, the inhibition of protein expression appears to be target specific. Neither molecule showed significant inhibition of VCAM-1 expression.

To understand the role of cholesterol in improving the function of ISIS-3082, we asked whether this molecule works merely because it is more hydrophobic than an unconjugated oligonucleotide or through specific protein-mediated (e.g., apo-E) binding and entry into cells. The answer was obtained by synthesizing and analyzing other lipophilic conjugates of ISIS-3082. Adamantane, pyrene, eicosenoic acid, and $C_{16}$-glyceride lipid nucleoside conjugates were synthesized and incorporated into ISIS-3082 in the same fashion as cholesterol. Similar lipophilic molecules have been conjugated to oligonucleotide and studied in an HIV system (16).

A reverse-phase HPLC assay was used to measure the relative lipophilicities of these conjugates as a model for the interaction between the cell membrane and the antisense oligonucleotide. The retention time of the oligonucleotide (and

**Table 1** ICAM-1 Oligonucleotides with Lipophilic Modifications

| Compound | Composition |
|---|---|
| ISIS-3082 | 5′-Ts Gs Cs As Ts sCs Cs Cs Cs Cs As Gs Gs Cs Cs As Cs Cs As T |
| ISIS-9047 | 5′-T*s Gs Cs As Ts Cs Cs Cs Cs Cs As Gs Gs Cs Cs As Cs Cs As T<br>(T* = 5′-octadecylaminothymidine) |
| ISIS-8005 | 5′-U* s Gs Cs As Ts Cs Cs Cs Cs Cs As Gs Gs Cs Cs As Cs Cs As T<br>(U* = 5′-(2′-*O*-hexylamino-carbonyl-oxycholesterol)-uridine |
| ISIS-9388 | 5′-T s Gs Cs As Ts Cs Cs Cs Cs Cs As Gs Gs Cs Cs As Cs Cs As U*<br>(U* = 5′-(3′-*O*-hexylamino-carbonyl-oxycholesterol)-uridine |



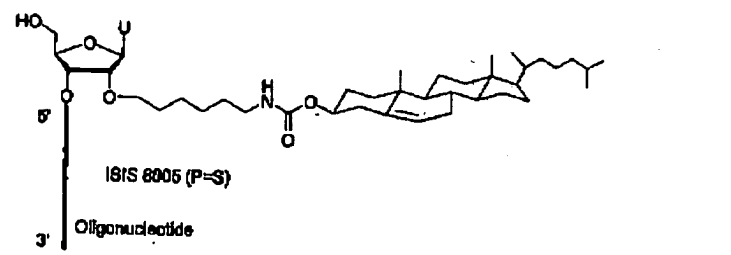
presumably the lipophilicity) increases with the number of carbon atoms in the pendant group. There is a linear correlation between the percentage of acetonitrile needed for elution and the total number of carbons. The two compounds having the same number of carbons (pyrene and eicosenoic acid) elute at the same time, while the group having the greatest number of carbons (glyceride lipid) has the longest retention time. Thus a wide spectrum of lipophilicites was observed from the least lipophilic, unconjugated ISIS 3082, to the glyceride lipid conjugate, ISIS 11826. In the antisense efficacy assays, without any added cationic lipid formulation, relative order of lipophilicity was not reflected in efficacy. While the cholesterol conjugate does inhibit ICAM-1 expression, other conjugates failed to inhibit ICAM-1 expression within the concentration range of 1–10 μM of oligonucleotides. The cholesterol-conjugated oligonucleotide shows a linear dose-dependent response in controlling the ICAM-1 expression. This experiment suggests that a receptor-mediated process may be operating in the case of cholesterol-conjugated oligonucleotides.

## 4. Pharmacokinetics of Cholesterol Conjugates and Other Lipophilic Conjugates

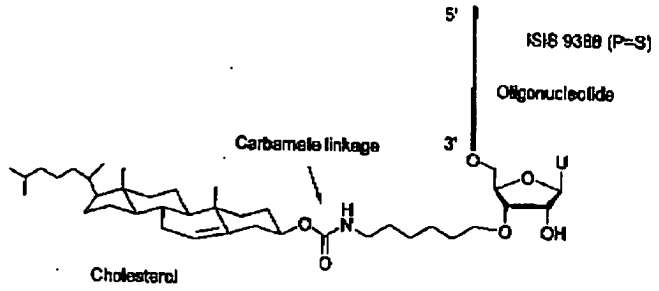
Biophysical and pharmacokinetic properties of lipophilic analogs of ISIS-3082 listed in Table 1 have been evaluated and reported (17). Compared to the parent compound, ISIS-3082, the three analogs (Fig. 5) with lipophilic conjugates, ISIS-9047 (5′-octadecylamine), ISIS-8005[5′-(2′-*O*-hexylamino-carbonyl-oxycholesterol)], and ISIS-9388 [3′-(3′-*O*-hexylamino-carbonyl-oxycholesterol)] were more lipophilic than ISIS-3082 (three- and sevenfold, respectively, for the first two compounds as measured by reverse-phase HPLC retention times) but had similar binding affinity for complementary RNA (measured by thermal melting analysis, $T_m$).

Tissue distribution and half-life in mice were analyzed using radioatively labeled phosphorothioate ISIS-3082 and cholesterol and $C_{18}$ amine analogs. After bolus intraveneous injection, the initial volumes of distribution of these more lipophilic phosphorothioate analogs, ISIS-9047 and ISIS-8005, were less and the initial clearance from plasma was slower than was that of ISIS-3082. ISIS-3082 distributes mainly to liver and kidney. Conjugation to cholesterol (ISIS-8005) or to $C_{18}$ amine (ISIS-9047) increased substantially the fraction of the dose accumulated by the liver. Both also had a somewhat longer retention in plasma than ISIS-3082. However, neither lipophilic conjugate had an effect on metabolite patterns in plasma, liver, or kidney compared to ISIS-3082.

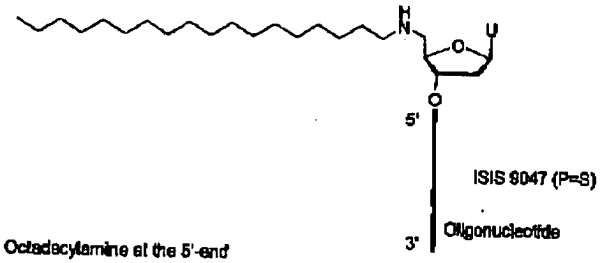
As a model to relative protein binding to human serum albumin, binding constants to bovin serum albumin (BSA) were measured. Binding to serum proteins plays a key role in the pharmacokinetics of oligonucleotides and, in view of the effects of phosphorothioates on clotting and complement activation, their




Cholesterol at the 5'-end




Cholesterol at the 3'-end




Octadecylamine at the 5'-end

**Figure 5** Isis lipophilic conjugates described in Table 1.



toxicological properties as well. As a model for protein binding to human serum albumin in plasma, binding constants to BSA were measued. Binding of ISIS-3082 to BSA was comparable to that observed for other phosphorothioate oligodeoxynucleotides (17). Binding was salt-dependent and, at physiological salt concentrations, the $K_d$ was approximately 140 μM. The affinities of the lipophlic conjugates, ISIS-8005 and ISIS-9047, were greater at physiological salt concentrations than the affinity of ISIS-3082. Experiments in which ISIS-3082, ISIS-9047, and ISIS-8005 were coincubated confirmed the lack of salt dependency of binding of the two analogs and the salt dependency of binding ISIS-3082 to BSA. These data and other data suggest that phosphorothioate linkages are necessary for binding to BSA under physological conditions. and that increased lipophilicity, either throughout the molecule or at the 5′-terminus, increased binding at physiological salt concentrations. Thus, more lipophilic phosphorothioate-containing analogs may bind to more than one type of site in BSA or more tightly to the phosphorothioate site.

The differences in serum protein binding are reflected in the pharmacokinetics of the analogs. The 5′-cholesterol adduct (ISIS-8005) and the $C_{18}$ amine conjugate (ISIS-9047) both showed increased retention in plasma relative to ISIS-302. Both also increased the proportion of dose in the liver substantially compared to ISIS-3082. It is not clear whether this change is due to an active transport of the lipophilic conjugates into the liver or whether the effects observed were simply due to the changes in lipophilicity. However, there was no improvement in distribution to central nervous system.

Neither the 5′-cholesterol nor $C_{18}$ amine modification enhanced resistance to metabolism significantly compared to ISIS-3082 when oligonucleotide was analyzed after extraction from liver of treated mice. However, the 3′-cholesterol conjugate of ISIS 3082 (ISIS-9388) was much more stable than the 5′-conjugate. The 3′-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS-9388.

The 3′-cholesterol analog (ISIS-9388) was evaluated for its binding to lipoproteins and its biodistribution (18). ISIS-9388 associated with lipoproteins and had an altered metabolic fate compared with the nonconjugated phosphorothioate oligonucleotide ISIS-3082. The lipoprotein-associated oligonucleotide is not rapidly filtered by the kidneys and probably does not leak as rapidly in peripheral tissues as the underivatized oligonucleotide. As a result, ISIS-9388 circulates longer, which allows a longer exposure to its target.

## 5. In Vivo Therapeutic Efficacy of Cholesterol-Conjugated ICAM-1 Oligonucleotides

The greater concentration in liver was correlated with the therapeutic effect of the ISIS-8005 as measured by ICAM-1 mRNA levels in mouse liver in vivo. In

lipopolysaccharide-induced expression of ICAM-1 mRNA by intravenous treatment of the mouse with ISIS 8005 at a dose of 10 mg/kg 24 h and 2 h prior to polysaccharide treatment, improved efficacy of the drug was observed presumably due to cholesterol conjugation, as indicated by mouse ICAM-1 RNA levels in the liver. At this concentration, the unmodified oligonucleotide ISIS-3082 does not have any effect.

## 6. Evaluation of Cholesterol-Conjugated Antisense Oligonucleotides in Other Biological Targets

Following the methods used to synthesize 2′- and 3′-cholesterol-uridine conjugates (15,19), the chemistry was extended to other nucleosides (adenosine and cytosine) and antisense oligonucleotide conjugates for several disease targets were synthesized. Synthesis of these cholesterol nucleosides was carried out by condensing cholesterol chloroformate with 2′-*O*-alkylamine or 3′-*O*-alkylamine of the appropriate nucleoside. The 2′-*O*-alkylamines were derived from direct alkylation procedure (20).

The 3′-cholesterol conjugated cytosine CPG was incorporated into an Ha-*ras* antisense oligonucleotide ISIS-13748 (the conjugate is the analog of 2′-deoxy-oligonucleotide phosphorothioate ISIS-2570). This compound was evaluated to determine the effect of cholesterol conjugation on RNase H activity in a cell-free assay. The cholesterol conjugate did not affect the RNase H cleavage rates or the extent of cleavage of the target RNA (Lima and Crooke, unpublished results, Isis Pharmaceuticals).

Activity of cholesterol-conjugated 2′-deoxy and 2′-*O*-MOE gapmer phosphorothioate oligonucleotides targeted against PKC-α and C-*raf* mRNAs has been reported (21). ISIS-8006, the cholesterol conjugate, was as active as the phosphorothioate oligonucleotide, ISIS-5132, in the presence of cationic lipids. In cultured T24 cells, in the absence of cationic lipids, ISIS-8006 was able to inhibit C-*raf* kinase mRNA expression while ISIS-5132 was inactive at 5-μM concentration. In the same experiment, cholesterol-conjugated ICAM-1 antisense oligonucleotide was inactive in inhibiting C-*raf* kinase, supporting an antisense mechanism of action.

Cholesterol analogs of an antisense oligonucleotide targeting PKC-α have also been evaluated. ISIS-3521 is a potent, selective inhibitor of PKC-α gene expression in cell culture, has been shown to inhibit tumor growth in mice (22), and is currently in Phase III clinical trials. Three cholesterol analogs listed in Table 2 were tested in A549 cells and in T24 cells at 10-μM concentration without cationic lipids. The cholesterol analogs were able to reduce PKC-α mRNA levels in both cell lines while ISIS-3521 was inactive.



**Table 2** Human C-*raf*, PKC-α and H-*ras* Oligonucleotides and Gapmers and Their Cholesterol Conjugates

| Compound | Sequence | Target | Chemistry |
|---|---|---|---|
| ISIS-2570 | CCACACCGACGGCGCCC | Human H-ras | 2′-H/P=S |
| ISIS-13748 | CCACACCGACGGCGCCC* | Human H-*ras* | 2′-H/P=S with 3′-cholesterol |
| ISIS-5132 | TCCCGCCTGTGACATGCATT | Human c-*raf* | 2′-H/P=S |
| ISIS-8006 | U*CCCGCCTGTGACATGCATT | Human c-*raf* | 2′-H/P=S and 5′-cholesterol |
| ISIS-3521 | GTT CTC GCT GGT GAG TTT CA | Human PKC-α | 2′-H/P=S |
| ISIS-8007 | GU*T CTC GCT GGT GAG TTT CA | Human PKC-α | 2′-H/P=S |
| ISIS-9520 | U*GTT CTC GCT GGT GAG TTT CA | Human PKC-α | 2′-H/P=S and 5′-Cholesterol |
| ISIS-12373 | GTT CTC GCT GGT GAG TTT CA U* | Human PKC-α | 2′-H/P=S and 3′-Cholesterol |
| ISIS-9531 | *GUU CUC GCT GGT GA GUU UCA U* | Human PKC-α | P=S gapmer; 2′-F in wings |
| ISIS-9533 | *GUU CUC GCT GGT GA GUU UCA U** | Human PKC-α | P=S gapmer; 2′-F in wings and 3′-cholesterol |

## 7. Effect of Cholesterol Conjugation: Reports from Other Laboratories

Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5′ cholesterol-conjugated phosphorothioate antisense oligonucleotides has been reported (23). Multiple drug resistance (MDR) is a result of overexpression of the P-glycoprotein drug transporter, a product of the MDR1 gene, and is a significant problem in cancer therapeutics. It was shown that 2′-deoxy phosphorothioate antisense oligonucleotides reduce levels of MDR1 message, inhibit expression of P-glycoprotein, and affect drug uptake in MDR mouse 3T3 fibroblasts. An oligonucleotide (ISIS-5995) directed against a sequence overlapping the AUG start codon was effective in reducing MDR1 transcript and protein levels when used at submicromolar concentrations in conjunction with cationic lipids, whereas a scrambled control oligonucleotide (ISIS-10221) was ineffective. Substantial and specific antisense effects could also be attained with a 5′ cholesterol conjugate of the ISIS-5995 sequence without the need for cationic lipids. The 5′ cholesterol ISIS-5995, but the not 5′ cholesterol ISIS-10221, reduced MDR1 message and P-glycoprotein levels by 50–60% when used at 1-μM concentrations. In parallel, treatment with 5′ cholesterol ISIS-5995 also enhanced cellular accumulation of rhodamine 123, a well-known substrate of the P-glycoprotein transporter. The effectiveness of the cholesterol-conjugated ISIS-5995 appears to be due to its rapid and increased cellular uptake as compared to unconjugated oligonucleotide, as indicated in flow cytometry and confocal microscopy studies.

The pharmacokinetics of cholesterol conjugated oligonucleotides with unconjugated phosphorothioate oligonucleotides in female mice has been reported also by the researchers at Genta. They also observed that conjugation of cholesterol to phosphorothioate oligonucleotides increased the plasma half-life (24). Sixty minutes after injection, the levels of 3′-cholesterol conjugates are 3.8 times higher than those of unconjugated oligonucleotide, while the levels of 5′- and 5′,3′-cholesterol conjugated oligonucleotides are 7.4 times higher.

Cholesterol conjugation has also been studied by Iverson et al. (25). 5′-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides with sequence complementary to the rat CYP2B1 mRNA were evaluated in adult male Sprague-Dawley rats for their pharmacokinetic properties and ability to modulate CYP2B1 expression in vivo. After intraperitoneal administration of $^{35}$S-labeled oligodeoxynucleotides, volume of distribution for the phosphorothioate was reduced to 33% for the 5′-cholesteryl-conjugate oligodeoxynucleotide and the elimination half-life was reduced 50% for the cholesteryl-modified oligodeoxynucleotide relative to unconjugated controls. Hexobarbital sleep times, a measure of CYP2B1 enzyme activity in vivo, increased nearly 30% in cholesterol oligodeoxynucleotide–treated animals.



Alefelder et al. reported the introduction of 3′- and 5′-terminal phosphorothioates into oligonucleotides and their postsynthetic modification with α-(bromoacetamido)-3-cholesterol and 2-(5′-nitropyridyl)-3-cholesterol disulfide to give cholesterol conjugates (26). A similar approach was used by Zhang et al. based on a phosphoramidite intermediate (27). The phosphorothioate derivatives with cholesterol at the 3′-end exhibit potent anti-HCMV activity, enhanced nuclease resistance and cellular association. An H-phosphonothioate solid-phase synthesis method facilitated the synthesis of oligonucleotide conjugates, as demonstrated by the example of attachment of 5′-cholesterol oligonucleotides to phosphorodithioates (28). Acetal-mediated cholesterol conjugation has been reported by Pfleiderer's group (29). The 5′-*O*- or 2′-*O*-position of appropriately protected thymidine or uridine was subjected to acid-catalyzed reaction with cholesterylvinylether (29). The corresponding cholesteryl-acetals were derivatized to the phosphoramidites or succinates attached to polystyrene as solid support.

The effects of conjugating cholesterol to either or both ends of a phosphorothioate oligonucleotide were analyzed in terms of cellular uptake and antisense efficacy against the p75 nerve growth factor receptor (p75) in differentiated PC12 cells, which express high levels of this protein (30). The addition of a single cholesteryl group to the 5′ end significantly increased cellular uptake and improved p75 mRNA down-regulation compared with the unmodified oligonucleotide. The 3′-cholesterol analog was more active still. Bis-cholesteryl (5′- and 3′-) conjugated oligonucleotide was even more potent and at 1 μM as effective as high concentrations of cycloheximide at decreasing synthesis of p75. Inhibitory effects on the multiplication of mouse hepatitis virus by cholesterol-modified oligonucleotides complementary to the leader RNA have also been reported (31).

Cellular uptake of 3′-cholesterol-conjugated oligonucleotides has been examined with a real-time confocal laser microscopy (32). Cytosolic uptake of cholesterol conjugate was five times as rapid as that of phosphorothioate oligonucleotides and nuclear uptake of cholesterol conjugate was twice as fast as that of unmodified oligonucleotide. In this study, oligonucleotides were also labeled with 5′-fluorescein and the effect of fluorescein on uptake has not been separated from the effect of cholesterol.

Inhibition of transactivation of human immunodeficiency virus type-1 (HIV-1)-LTR by cholesterol-conjugated antisense oligonucleotides was compared to that of their unconjugated analogs in vitro (33) to study the efficiency of antisense oligonucleotides in inhibiting LTR-(HIV-1)-directed CAT expression catalyzed by tat protein. Antisense oligonucleotides modified by conjugation of cholesterol at the 3′ end have a severalfold higher inhibitory response, and the inhibition by antisense oligonucleotides is sequence-specific.

In addition to effects on cellular uptake, cholesterol modulates oligonucleotide-mRNA hybrid stability via hydrophobic interactions (34). Two series of 3′-cholesterol- and/or 5′-cholesterol-conjugated oligonucleotides have been synthe-